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The Stroma of Higher Plant Plastids Contain ClpP and ClpC, Functional Homologs of *Escherichia coli* ClpP and ClpA: An Archetypal Two-Component ATP-Dependent Protease

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A cDNA representing the plastid-encoded homolog of the prokaryotic ATP-dependent protease ClpP was amplified by reverse transcription–polymerase chain reaction, cloned, and sequenced. ClpP and a previously isolated cDNA designated ClpC, encoding an ATPase related to proteins encoded by the *ClpA/B* gene family, were expressed in *Escherichia coli*. Antibodies directed against these recombinant proteins recognized proteins in a wide variety of organisms. N-terminal analysis of the Clp protein isolated from crude leaf extracts showed that the N-terminal methionine is absent from ClpP and that the transit peptide is cleaved from ClpC. A combination of chloroplast subfractionation and immunolocalization showed that in Arabidopsis, ClpP and ClpC localize to the stroma of the plastid. Immunoblot analyses indicated that ClpP and ClpC are constitutively expressed in all tissues of Arabidopsis at levels equivalent to those of *E. coli* ClpP and ClpA. ClpP, immunopurified from tobacco extracts, hydrolyzed *N*-succinyl-Leu-Tyr-amidomethylcoumarin, a substrate of *E. coli* ClpP. Purified recombinant ClpC facilitated the degradation of ^{3}H-methylcasein by *E. coli* ClpP in an ATP-dependent fashion. This demonstrates that ClpC is a functional homolog of *E. coli* ClpA and not of ClpB or ClpX. These data represent the only in vitro demonstration of the activity of a specific ATP-dependent chloroplast protease reported to date.

INTRODUCTION

In all organisms, proteins within the same subcellular compartment have half-lives that can differ by up to three orders of magnitude, implying that their turnover is a highly regulated, selective process (Gottesman and Maurizi, 1992). Peptide hydrolysis is exergonic; therefore, it is surprising that up to 80% of protein degradation in *Escherichia coli* requires ATP hydrolysis (Maurizi, 1992). However, this coupling of proteolysis to ATP hydrolysis is emerging as a general principle for both prokaryotes and eukaryotes (Maurizi, 1992). In eukaryotes, the major energy-dependent protease system of the cytosol is the ubiquitin system (Jentsch, 1992), which degrades proteins by first coupling a small protein, ubiquitin, to the protein destined for degradation (Shanklin et al., 1987); a multisubunit protease (also referred to as the proteasome) then degrades the tagged protein (reviewed in Ciechanover, 1994). ATP hydrolysis is required for both the coupling of ubiquitin to the target protein and the degradation by the proteasome. In prokaryotes, the two archetypical ATP-dependent proteases are La and ClpP (the latter is also referred to as Ti); they are characterized by their ability to hydrolyze casein in vitro in an ATP-dependent fashion.

Approximately half of the protein in plants resides within plastids, but relatively little is known about their degradation. Hammond and Preiss (1983) first reported ATP-dependent protease activity in higher plants. To assay the activity, they used ^{14}C-casein as a substrate and found that it would cause ATP-dependent release of trichloroacetic acid (TCA)–soluble radioactivity from this substrate. Subsequently, two reports were published demonstrating ATP-dependent proteolytic activity in isolated chloroplasts (Liu and Jagendorf, 1984; Malek et al., 1984). The results demonstrated that when isolated plastids were fed labeled amino acids, ~30% of the newly synthesized proteins were degraded in an energy-dependent manner during a subsequent brief chase. Because synthesis of chloroplast proteins occurred in isolated organelles, the newly synthesized protein subunits were expressed in the absence of their cytoplasmically synthesized counterparts. In subsequent in vivo labeling studies, Liu and Jagendorf (1985) showed that the same proportion of newly synthesized chloroplast proteins were degraded in the absence of cytoplasmic synthesis, but the majority of these proteins were not degraded when cytoplasmic synthesis occurred. The energy-dependent plastid protease responsible for degrading such newly synthesized but unassembled chloroplast polypeptides remains to be identified. One candidate was the ubiquitin system, because several groups reported ubiquitin conjugation activity in chloroplast extracts (Wettern et al., 1990; Veierskov and Ferguson, 1991).

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A more rigorous analysis by Beers et al. (1992) showed that although low levels of ubiquitin-conjugated proteins are present in plastids, the ubiquitin system itself is not present in this compartment. Another candidate for ATP-dependent plastid proteolysis is the Clp protease system.

The Clp system was first identified in *lon*⁻ *E. coli*; it is composed of two nonhomologous components, a protease and an ATPase that is required to present substrates to the protease facilitating their degradation (Hwang et al., 1987; Katayama-Fujimura et al., 1987). Two distinct ATPases that have been identified are ClpA (83 kD) and ClpX (46 kD); they can facilitate the degradation of different substrates in conjunction with the 21-kD proteolytic component ClpP (Hwang et al., 1987; Katayama-Fujimura et al., 1987; Wojtkowiak et al., 1993). The Clp protease does not form a stable proteolytic complex; instead, ClpP and the presenting subunits, ClpA or ClpX, exist as homooligomers, which associate transiently to degrade proteins (Maurizi, 1991). The active protease, ClpAP, is composed of four rings of six or seven identical subunits comprising a $P_{(14)}$, $A_{(12)}$ holoenzyme (Flanagan et al., 1995; Kessel et al., 1995).

E. coli ClpP was cloned by Maurizi et al. (1990a), who showed that it encodes a previously undescribed class of serine proteases. Homology searches revealed that *E. coli* ClpP is 42% identical to the deduced amino acid sequence of the tobacco plastid genome open reading frame ORF196 (Maurizi et al., 1990b), suggesting that plastids may express a Clp-like protease. Recently, nuclear-encoded ClpPs with putative chloroplast transit peptides were identified in tomato and Arabidopsis (Schaller and Ryan, 1995; J. Shanklin, unpublished results).

ClpA was the first identified ATPase able to facilite proteolysis by ClpP (Hwang et al., 1987; Katayama-Fujimura et al., 1987). *ClpA* belongs to a family of related genes encoding ClpAs, ClpBs, and ClpCs; these proteins are ATPases that contain two nonhomologous nucleotide binding domains. They differ from each other in terms of the length of the spacer region separating the two domains. In *E. coli*, the linkers are five and 124 residues for ClpA and ClpB, respectively; ClpC has an intermediate length linker of ~60 residues (Gottesman et al., 1990; Squires and Squires, 1992). The genes encoding these proteins are widespread in occurrence: ClpA proteins are found in bacteria; ClpB proteins are found in bacteria, yeast, trypanosomes, mammals, and plants; and ClpC proteins are found in bacteria and plants (summarized in Gottesman et al., 1990; Squires and Squires, 1992; and Lee et al., 1994; Schirmer et al., 1994). Although ClpA interacts with the ClpP protease, ClpB acts independently of ClpP to convey thermotolerance by a mechanism that remains to be determined (reviewed in Parsell and Lindquist, 1993). Cytoplasmically localized plant ClpB homologs recently were shown to be functionally homologous to yeast ClpB (Lee et al., 1994; Schirmer et al., 1994). In contrast, the function of ClpC is not known; however, the identification of a chloroplast transit peptide sequence for plant ClpC sequences (Gottesman et al., 1990) and the uptake of ClpC into chloroplasts have shown that it is a plastid-targeted protein (Moore and Keegstra, 1993) and have led to speculation that it might interact with the plastid ClpP to play a role in protein degradation. However, a comparison of the sequence of plant ClpC with *E. coli ClpA* and *ClpB* genes has suggested that ClpC is more closely related to ClpB than ClpA (Gottesman et al., 1990).

We have begun to investigate the higher plant Clp protease by characterizing the localization and expression of Clp proteins in the model system Arabidopsis. Both ClpP and ClpC subunits are localized primarily in the stroma of Arabidopsis plastids and are constitutively expressed at approximately equimolar levels in all tissues under a variety of environmental conditions. To address the biochemical properties of plastid Clp proteins, we used tobacco ClpP and pea ClpC, because gene sequences were available. Here, we report that ClpP from tobacco is able to degrade a fluorogenic substrate cleaved by *E. coli* ClpP and that recombinant pea ClpC facilitates the degradation of methylcasein by *E. coli* ClpP.

RESULTS

Cloning of the Plant ClpP cDNA, Expression of ClpP and ClpC in *E. coli*, and Production of Monospecific Antibodies

The existence of ClpP in plastids of higher plants was inferred from the observations that all plant plastid genomes sequenced to date have an ORF homologous to bacterial ClpP and that a series of weakly immunoreactive bands are seen in plant extracts probed with anti–*E. coli* ClpP antibodies (Maurizi et al., 1990b). The tobacco ClpP homolog, encoded by plastid genome ORF196, is interrupted by two introns (Shinozaki et al., 1986). Reverse transcription–polymerase chain reaction using RNA extracted from tobacco chloroplast resulted in the amplification of a 591-bp cDNA encoding an appropriately processed mRNA, showing that this gene is expressed. The amplified fragment was cloned into a bacterial expression vector pET3a and sequenced to confirm its identity. This analysis defined intron splice sites that were previously reported incorrectly (Shinozaki et al., 1986; Maurizi et al., 1990b; ClpP cDNA, GenBank accession number U32397).

The previously identified ClpC ORF from a pea cDNA was amplified using polymerase chain reaction and cloned into the expression vector pET3a. Plasmids encoding tobacco ClpP or pea ClpC were transformed into *E. coli* BL21(DE3) cell lines deficient in ClpP or ClpA, respectively. Expression of the plant Clp homologs resulted in the accumulation of proteins with predicted molecular masses of ~23 kD for ClpP and 100 kD for ClpC (Figure 1). Because ClpP and ClpC accumulated as insoluble inclusion bodies in *E. coli*, we could purify them by using repeated extractions with the nonionic detergent Triton X-100, followed by solubilization and back-extraction from guanidine-HCl. This procedure resulted in highly enriched polypeptides that we used to produce antibodies (Figure 1). The antibodies were purified using the corresponding immobilized antigen and were determined to be monospecific based on

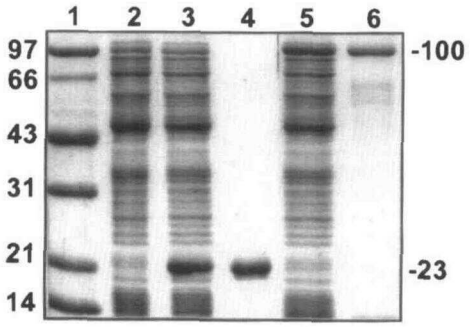

Figure 1. Expression of Plant Clp Homologs in *E. coli*.

An 11% Coomassie Brilliant Blue R 250–stained gel. Lane 1 contains the molecular mass markers; lanes 2, 3, and 5 contain 50 μg of total protein from induced cells containing plasmids pET3a alone, pClpP, and pClpCtot, respectively; and lanes 4 and 6 contain 3 μg of protein purified as described in the text. Numbers at left represent molecular masses in kilodaltons. The positions of the ClpC polypeptide at 100 kD and the ClpP polypeptide at 23 kD are indicated at right.

their immunoreactivity with crude extracts from higher plants (Figure 2).

Identification of Higher Plant ClpP- and ClpC-Immunoreactive Proteins

The expression of plant ClpP previously has been inferred from immunoblot analyses of spinach crude extract revealing a series *E. coli* ClpP-immunoreactive species (Maurizi et al., 1990b). However, the same antibody preparation failed to react with purified recombinant tobacco ClpP, suggesting that the proteins previously identified might not include ClpP (data not shown). Here, we report that immunopurified tobacco anti-ClpP antibodies strongly reacted with single proteins ranging in molecular masses from 23 to 26 kD in the dicots tobacco, pea, and Arabidopsis and in the monocot rice; however, they fail to detect any proteins in extracts from *Dunaliella salina* or *E. coli* (Figure 2A). Anti–*E. coli* ClpP antibodies generated in this study, like those made by M.R. Maurizi, reacted only with *E. coli* ClpP and not with plant ClpP proteins (Figure 2C). It is somewhat surprising that tobacco and *E. coli* ClpP proteins do not share immunological determinants, because they share 42% amino acid identity (67% identity plus similarity).

Anti–pea ClpC antibodies react with proteins in higher plants, algae, and *E. coli* (Figure 2B). The predominant bands at ~90 kD for plants and algae, and at ~85 kD for *E. coli* are consistent with the predicted molecular masses for plant ClpC proteins and *E. coli* ClpA.

ClpP and ClpC Are Constitutively Expressed in Arabidopsis

Both ClpP- and ClpC-immunoreactive species were seen in all (photosynthetic and nonphotosynthetic) tissues of Arabidopsis and at all times during development, suggesting that both ClpP and ClpC are expressed in a variety of types of plastids (Figures 3A and 3B). Quantitative analysis of similar immunoblots suggests that ClpP typically ranges in concentration from 0.01 to 0.03% of total protein, with the highest levels seen in expanding leaves and the lowest levels in senescing leaves. ClpC typically ranges from 0.05 to 0.15% of total protein (data not shown), with comparable levels in various tissues and at

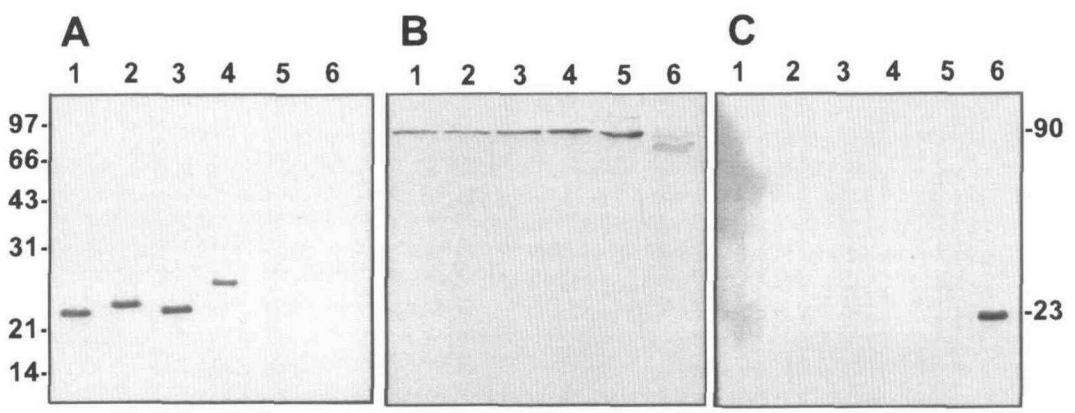

Figure 2. Clp-Immunoreactive Peptides Are Seen in Diverse Organisms.

Immunoblots of 50 μg of total protein extracts from various organisms were probed with immunopurified anti-Clp antibodies. Lanes 1 contain tobacco; lanes 2, pea; lanes 3, Arabidopsis; lanes 4, rice; lanes 5, *Dunaliella*; lanes 6, *E. coli*.

(A) Anti–tobacco ClpP antibody.

(B) Anti–pea ClpC antibody.

(C) Anti–*E. coli* ClpP antibody.

Numbers at left represent molecular masses in kilodaltons. The positions of the ClpC polypeptide at 90 kD and the ClpP polypeptide at 23 kD are indicated at right.

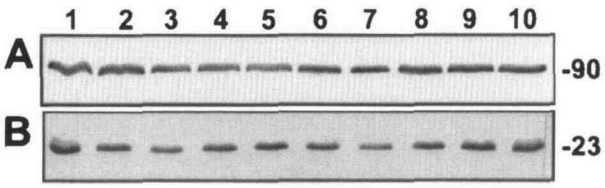

Figure 3. Plant ClpP and ClpC Are Constitutively Expressed.

Arabidopsis tissues (50 μg of crude extracts) were used for immunoblot analysis. Lane 1 contains expanding leaf extract; lane 2, mature leaf; lane 3, senescent leaf (stages S2 to S4); lane 4, petiole; lane 5, flower; lane 6, green silique at mid-stage of development; lane 7, root; lane 8, etiolated leaf; lane 9, heat-shocked leaf; and lane 10, dehydrated leaf.

(A) Anti-pea ClpC antibody.

(B) Anti-tobacco ClpP antibody.

The positions of the ClpC polypeptide at 90 kD and the ClpP polypeptide at 23 kD are indicated at right.

various times during development. Plant ClpP and ClpC are present in approximately equimolar amounts, and their expression levels are similar to those of *E. coli* (Hwang et al., 1988; Katayama et al., 1988). We saw no substantial differences in the levels of ClpP or ClpC following exposure to heat shock or dehydration, which are conditions shown to result in the accumulation of ClpB in *E. coli* (Maurizi, 1992) and ERD1 in Arabidopsis (Figure 3; Kiyosue et al., 1993), respectively.

Identification of ClpP and ClpC in the Stromal Fraction of Chloroplasts

To define the localization of Clp subunits, isolated chloroplasts were lysed and subfractionated by centrifugation, and the resulting fractions were probed using protein gel blot analysis (Figure 4). Effective separation of stromal and thylakoid fractions was demonstrated by the lack of contamination between the fractions containing the stromal marker stearoyl–acyl carrier protein desaturase and the thylakoid lumen marker plastocyanin (Figures 4C and 4D). Both ClpP and ClpC levels in the total soluble fraction were approximately equivalent to their levels in total unfractionated chloroplasts (Figures 4A and 4B, lanes 1 and 2). However, ClpC homologs have been isolated three times in screens designed to identify chloroplast envelope proteins (Moore and Keegstra, 1993; Ko et al., 1994; reported in Squires and Squires, 1992), suggesting that they may associate with the envelope and implying a possible role in the import/uptake of cytoplasmically synthesized proteins. Therefore, immunoelectron microscopy was performed on thinly sectioned Arabidopsis mesophyll cells to address the possibility that Clp proteins might localize to the chloroplast envelope. ClpP- and ClpC-immunoreactive proteins were detected only in chloroplasts (data not shown). There was no evidence for accumulation of Clp proteins at the chloroplast envelope; rather, ClpP and ClpC appeared to be homogeneously dispersed throughout the stroma (Figures 5A and 5B). The low abundance of the Clp proteins is reflected in their low-labeling density.

Plant ClpP and ClpC Have Activities Equivalent to *E. coli* ClpP and ClpA

The involvement of the plant Clp homologs in proteolysis was inferred from the existence of a *ClpP*-like gene in the chloroplast genome and a gene encoding a ClpA/B-like product with a chloroplast transit peptide (Gottesman et al., 1990). We therefore sought to obtain direct evidence for such involvement.

ClpP

To determine the biochemical activity of ClpP, it first was necessary to isolate the ClpP protein. Because recombinant tobacco ClpP was insoluble in *E. coli*, the protein was isolated from tobacco leaf extracts by immunoprecipitation. A 23-kD polypeptide was immunoprecipitated from tobacco leaf extract using immune but not preimmune antibodies (Figure 6A, lanes 3 and 4). Because Tanaka et al. (1989) reported cross-reactivity between anti–*E. coli* ClpP antibodies and components of the eukaryotic proteasome, we determined the N-terminal sequence of the immunopurified 23-kD protein to confirm its identity. The sequence PIGVPKVPFRSP corresponds to the N terminus of ClpP from which the terminal methionine had been removed. To determine whether the immunoprecipitated

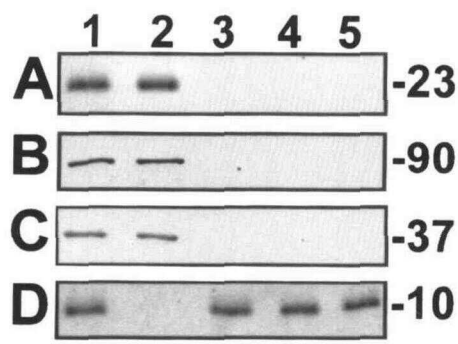

Figure 4. ClpP and ClpC Are Located in the Total Soluble Extra-Thylakoid Chloroplast.

Various fractions of Arabidopsis chloroplasts using 11% gels, 50 μg of total chloroplast extract, and equivalent proportions of subfractions were separated, loaded, and transferred to nitrocellulose. Lane 1 contains total chloroplast extract; lane 2, total soluble extract; lane 3, total thylakoid fraction; lane 4, washed thylakoid fraction; and lane 5, thylakoid lumen extract.

(A) Anti-tobacco ClpP antibody.

(B) Anti-pea ClpC antibody.

(C) Anti-avocado stearoyl-acyl carrier protein desaturase antibody.

(D) Anti-plastocyanin antibody.

Numbers at right indicate molecular masses in kilodaltons.

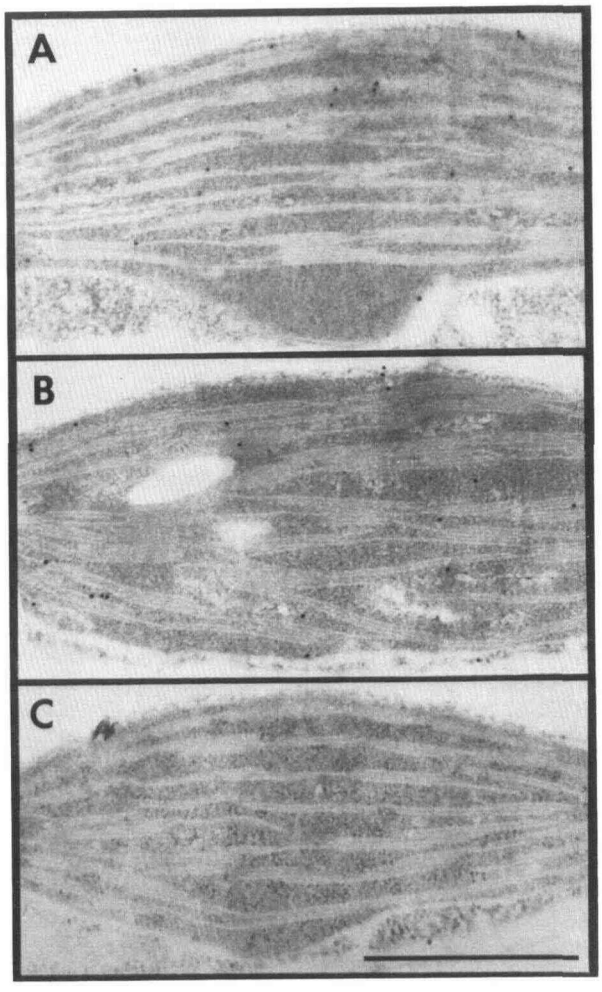

Figure 5. ClpP and ClpC Are Localized to the Stroma.

Thin sections of Arabidopsis chloroplasts were subjected to immunoblot analysis.
(A) Anti–tobacco ClpP antibody.
(B) Anti–pea ClpC antibody.
(C) Nonimmune control.
The bar in **(C)** = 0.5 μm for **(A)** to **(C)**.

tobacco ClpP was active, we took advantage of the observation of Woo et al. (1989). They determined that purified *E. coli* ClpP cannot degrade protein substrates in the absence of ATP but that ClpP alone can degrade the fluorogenic peptide *N*-succinyl-Leu-Tyr-amidomethylcoumarin. We added this substrate to resuspended immunoprecipitates of tobacco ClpP and monitored its degradation fluorometrically. Hydrolysis was seen with immune precipitate but not with preimmune precipitate, showing that tobacco ClpP is capable of degrading the same substrate as *E. coli* ClpP (Figure 6B). The lack of activity from control precipitates, in which the leaf extract was omitted, demonstrates that the observed ClpP activity did not originate from the immunopurification procedure. All subsequent efforts to recover functional ClpP from the antibody–ClpP complex were unsuccessful.

ClpC

When full-length ClpC was expressed in *E. coli*, it, like ClpP, resulted in the accumulation of an insoluble, inactive material. Because transit peptides can destabilize soluble proteins, we sought to express a mature ClpC in the hope that it would accumulate in a soluble, active form. The transit peptide

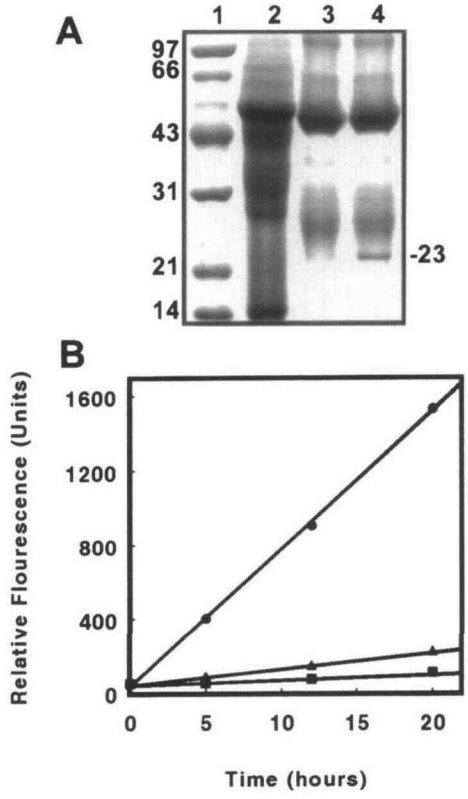

Figure 6. Immunopurified Tobacco ClpP Is Able To Hydrolyze *N*-Succinyl-Leu-Tyr-Amidomethylcoumarin.

(A) An 11% Coomassie blue–stained gel. Lane 1 contains molecular mass markers; lane 2, 50 μg of tobacco leaf lysate; lanes 3 and 4, immunoprecipitates of tobacco chloroplast extracts using preimmune and anti-ClpP antibodies, respectively. The position of the ClpP polypeptide is at 23 kD.
(B) Fluorogenic peptide degradation assay. Circles and triangles represent immunoprecipitates from tobacco chloroplast lysates using immune or preimmune antibodies, respectively; squares, control immunoprecipitate lacking chloroplast extract using immune antibodies. Degradation was monitored as the release of fluorescent coumarin from *N*-succinyl-Leu-Tyr-amidomethylcoumarin.

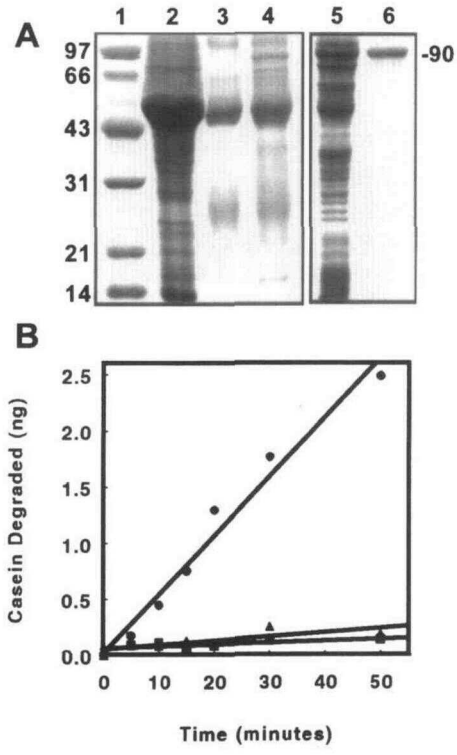

Figure 7. Purified Recombinant Pea ClpC Is Able To Facilitate ATP-Dependent ^{3}H-Methylcasein Degradation with *E. coli* ClpP.

(A) An 11% Coomassie blue–stained gel. Lane 1 contains molecular mass markers (given at left in kilodaltons); lane 2, 50 μg of pea crude lysate; lanes 3 and 4, immunoprecipitates of pea extracts using nonimmune and anti-ClpC antibody, respectively; lane 5, 50 μg of total extract of induced *E. coli* containing plasmid pClpCmat; and lane 6, 3 μg of purified ClpC. The position of the mature ClpC polypeptide is at 90 kD.

(B) ^{3}H-methylcasein degradation assay. Circles and triangles represent *E. coli* ClpP, with a saturating amount of purified pea ClpC, and in the presence and absence of ATP, respectively; squares, equivalent amount of purified ClpC in the absence of *E. coli* ClpP and in the presence of ATP.

cleavage site first had to be determined; therefore, we immunopurified mature ClpC from crude extracts of pea leaf for protein sequence analysis. A predominant band at 90 kD was present in the immune precipitate but not in the preimmune precipitate (Figure 7A, lanes 3 and 4). N-terminal analysis of this protein yielded the sequence MFERFTEKAIKVIM, which corresponds to residues 91 to 104 of the published sequence of ClpC (Moore and Keegstra, 1993). The four immediately preceding residues, IPRA, resemble the transit peptide cleavage consensus sequence V/I, X, A/C, A, described by Gavel and von Heijne (1990).

A second expression plasmid was constructed to express the mature pea ClpC in which Met-91 was used as the initiating methionine. The resulting soluble protein was purified by anion exchange chromatography followed by hydroxyapatite chromatography (Figure 7A, lanes 5 and 6). This purified recombinant ClpC was added to *E. coli* ClpP to assay for the degradation of ^{3}H-methylcasein (Figure 7B). Degradation depended on the addition of both ATP and ClpP. Attempts to reconstitute immunopurified plant ClpP with purified ClpC failed because conditions required to separate the antibody–ClpP complex caused the loss of catalytic activity in the fluorogenic peptide degradation assay. No caseinolytic activity was obtained when purified ClpC was added to undenatured immunoprecipitates, presumably because of steric hindrance resulting from antibody binding.

DISCUSSION

The proposal that plant ClpP and ClpC interact to form a plastid Clp-like protease was based on the identification of a ClpP homolog encoded by the plastid genome and a transit peptide at the N terminus of ClpC (Gottesman et al., 1990). The goal of this study was to define the biochemical function of these Clp-like proteins. Immunopurified tobacco ClpP hydrolyzed a fluorogenic peptide that is cleaved by *E. coli* ClpP, suggesting a similar proteolytic role for the plastid-localized protease. Purified recombinant ClpC facilitated the degradation of ^{3}H-methylcasein in conjunction with *E. coli* ClpP. This finding indicates that plant ClpC is a functional homolog of ClpA rather than ClpX or ClpB, because ClpX cannot facilitate the degradation of ^{3}H-methylcasein, and ClpB acts independently of ClpP. This work represents the only in vitro demonstration of the activity of a specific ATP-dependent chloroplast protease reported to date.

The function of the plastid Clp protease is not known. It is interesting that ClpC has been isolated as a chloroplast envelope–associated protein in at least three separate screens (reported in Squires and Squires, 1992; Moore and Keegstra, 1993; Ko et al., 1994), which has led to speculation that the protease may constitute part of the import/processing apparatus (Squires and Squires, 1992; Moore and Keegstra, 1993; Parsell and Lindquist, 1993). However, Moore and Keegstra (1993) also reported that the majority of ClpC was present in soluble extracts of chloroplasts. The fractionation data presented here are consistent with ClpP and ClpC being present in a soluble fraction outside the thylakoid membranes. Immunoelectron microscopy was performed to test the possibility that Clp proteins might somehow associate with the envelope. This did not appear to be the case; rather, both ClpP and ClpC are distributed throughout the chloroplast. We concluded that most of ClpP and ClpC are localized in the stroma of the chloroplasts and are therefore not likely to be involved in the import/processing process. Because plant ClpC binds to many surfaces, we included a low concentration of the nonionic detergent Triton X-100 in the extraction buffer to minimize losses due to nonspecific adsorption. This stickiness may explain the occurrence of ClpC in chloroplast envelope preparations.

ClpP homologs have been identified in all plastid genomes examined to date, even in the nonphotosynthesizing plant *Epifagus*, in which ClpP represents one of several remaining

functional genes (dePamphilis and Palmer, 1990). This suggests that it may fulfill some vital function in the plastid, because the dispensable plastid genes in this parasite have become nonfunctional through the accumulation of multiple mutations. Failure to generate tobacco cell lines with significantly reduced levels of ClpC by antisense expression is consistent with this hypothesis (J. Shanklin, unpublished results). Constitutive expression of Clp in plants suggests that it is involved in maintenance or housekeeping functions rather than a specific process such as photosynthesis or a developmental transition such as greening or senescence. The energy dependence of proteases has been proposed as a mechanism to convey selectivity to constitutively expressed proteases (Gottesman and Maurizi, 1992). Functions for such proteases include degradation of biosynthetic errors and misfolded or damaged proteins. For example, the Clp protease in *E. coli* has been shown to degrade ~15% of newly synthesized proteins containing amino acid analogs (Katayama et al., 1988). Squires and Squires (1992) speculated that ClpA may function as a chaperone protein that binds denatured proteins and may either facilitate refolding or present unfolded peptides to ClpP for degradation. Evidence for a link between the folding and degradation pathways recently was provided by Wickner et al. (1994), who showed that ClpA can act as a molecular chaperone like DnaK and DnaJ in activating the plasmid P1 RepA replication-initiator protein. Furthermore, RepA was a substrate for Clp protease, demonstrating for the first time a direct link between a chaperone activity and protein degradation.

A second link between folding and degradation was provided by Kandror et al. (1994), who reported that abnormal proteins in *E. coli* could be degraded in a process requiring ClpP and the molecular chaperone GroEL, using a mechanism independent of ClpA, ClpB, and ClpX. Clp also may play a role in degrading a specific set of short-lived proteins because it has been shown to mediate the N-end rule in prokaryotes by degrading proteins possessing destabilizing N-terminal residues; these proteins are degraded by the ubiquitin system in eukaryotes (Bachmair et al., 1986; Tobias et al., 1991). The major energy-dependent protease pathway in the cytosol or "eukaryotic compartment" in plants is the ubiquitin system (Vierstra, 1993), but this system is absent from the plastid or "prokaryotic compartment" (Beers et al., 1992) in which the Clp protease is now shown to be functional.

Plastids are composed of many multisubunit complexes with components that are synthesized in different compartments. For example, the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase is synthesized in the chloroplast, whereas the small subunit is synthesized in the cytoplasm and post-translationally transported into the chloroplast. It is well documented that in the absence of synthesis of the large subunit, the small subunit is rapidly degraded (Schmidt and Mishkind, 1983; Spreitzer et al., 1985). This represents a post-translational mechanism for fine tuning the protein synthesis from two compartments, presumably to avoid the buildup of unpaired and, therefore, inactive subunits. Liu and Jagendorf (1985, 1986) showed that turnover of unassembled subunits is ATP dependent; 7% of chloroplast proteins synthesized under normal conditions in the leaf are rapidly degraded, whereas in the absence of cytoplasmic protein synthesis, 25% of the newly synthesized proteins are rapidly degraded in an ATP-dependent fashion. As the only ATP-dependent chloroplast protease characterized to date, Clp is a candidate for mediating the degradation of such unpaired subunits.

In *E. coli*, ClpX, an ATPase distinct from ClpA/B-like ATPases, presents a separate set of substrates to ClpP (Wojtkowiak et al., 1993). It differs from ClpA/B ATPases in that it has one nucleotide binding domain rather than two. This suggests that the selectivity of ClpP can be modulated by altering the proportions of regulatory subunits in a manner similar to that described for the eukaryotic proteasome (Rechsteiner et al., 1993; Goldberg 1995). In addition to ClpC, two such potential regulatory subunits for ClpP have been identified in Arabidopsis. The first, ERD1, was isolated as a drought-induced cDNA (Kiyosue et al., 1993) and has the structure of a ClpA/B-like peptide with an N-terminal extension that probably is a transit peptide. The second potential regulatory subunit is a newly identified cDNA, with similarity to *E. coli* ClpX, that is the subject of ongoing investigation in this laboratory.

In summary, plastids have a functioning Clp protease system composed of ClpP, ClpC, and perhaps ClpX and ERD1. Now that the expression, localization, and function of the ClpP–ClpC system have been characterized, the important work of understanding its physiological role(s) can proceed.

METHODS

Materials

Pea Progress 9 and *Arabidopsis thaliana* (ecotype Columbia) were grown at 18°C in controlled environments under 250 μmol m^{-2} sec^{-1} of photosynthetically active radiation under 12/12- and 16/8-hr light/dark cycles, respectively. Tobacco MSU31 was grown in the greenhouse with supplemental light to give 16/8-hr light/dark photoperiods. For immunolocalization experiments, Arabidopsis was grown in Petri plates on media containing 1 × Murashige and Skoog salts (Sigma) and 290 mM sucrose under continuous illumination at 19°C.

Samples for Tissues and Environmental Effects

Arabidopsis seedlings that were 21 days old were heat shocked by gradually increasing the temperature to 38°C over 5 hr and maintaining it at 38°C for 4 hr (Schirmer et al., 1994) or they were deprived of water for 10 days, at which times the tissues were harvested. Leaf senescence was classified according to Lohman et al. (1994). To prepare extracts of Arabidopsis, various tissues were harvested, ground under liquid N_2, and extracted into 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8.0. Protein concentrations were determined using a Bradford assay kit (Bio-Rad) with BSA as standard, and a volume equivalent to 50 μg was brought to 5% (w/v) with trichloroacetic (TCA) acid. The precipitated protein was collected by centrifugation at 13,000*g* for 2 min, solubilized in 5 μL of 1 M Tris base, and prepared for electrophoresis by adding 5 μL of 2 × Laemmli loading buffer (Laemmli, 1970) and heating at 75°C for 2 min.

Cloning and Expression of Clp cDNAs

For reverse transcription–polymerase chain reaction of ClpP, total RNA was isolated from tobacco chloroplast using a guanidine isothiocyanate procedure (Logemann et al., 1987). First strand cDNA was synthesized using a SuperscriptII kit (Bethesda Research Laboratories) in conjunction with the 3′ oligonucleotide AATGGATCCTCATTCAACCGCTACAAGA. The cDNA then was amplified using polymerase chain reaction and the 5′ oligonucleotide GATGGATCCCATATGCCTATTGGTGTTCCAA in conjunction with the reverse transcription 3′ oligonucleotide described above. The resulting fragment was cloned into the pCRII vector (Invitrogen, San Diego, CA) and sequenced using dideoxynucleotide terminators (Sambrook et al., 1989). An NdeI-BamHI fragment encoding the complete reading frame of the ClpP cDNA was cloned into the corresponding sites in the expression vector pET3a, resulting in pClpP. The ClpC cDNA from pea (Moore and Keegstra, 1993) was amplified using either of the following 5′ oligonucleotides: TATACATATGGCTAGAGTTTTGGCT (for the full peptide) or TATACATATGTTTGAGCGTTTCACC (for the mature peptide), in conjunction with a 3′ oligonucleotide, TATACATATGTTATATGGAAAGAGCCTC.

The resulting fragments were restricted with NdeI and cloned into the NdeI site of expression plasmid pET3a, resulting in pClpCtot and pClpCmat. A 2376-bp BamHI fragment encoding the entire 3′ region of the pClp cDNA was used to replace the corresponding fragment of pClpCmat. The remaining 5′ 542-bp NdeI-BamHI fragment was sequenced using dideoxynucleotide terminators to ensure that the sequence was free from secondary mutations. All pET clones were introduced into BL21(DE3) derivatives for expression of recombinant protein according to Studier et al. (1990). Tobacco ClpP was expressed in strain SG1146 (BL21[DE3] Δ*ClpP*), and pea ClpC constructs were expressed in SG1147 (BL21[DE3] Δ*ClpA*).

Protein Purification, Antibody Production, and Immunological Procedures

Both pClpP and pClpCtot resulted in the production of an insoluble polypeptide in *Escherichia coli*. They were purified by lysing the cells with a French press in 100 mM sodium phosphate, 1 mM EDTA, 0.1% (w/v) Triton X-100, pH 7.5. The insoluble fraction was collected by centrifugation at 10,000*g* for 5 min, washed four times in lysis buffer containing 2% (w/v) Triton X-100, collected by centrifugation, and then resuspended in 100 mM sodium phosphate, pH 7.5. Guanidine-HCl was added to 8 M to solubilize the protein, followed by back extraction into 100 mM sodium phosphate, pH 7.5, and collection by centrifugation as before. This protein was resuspended and used to inoculate rabbits to raise polyclonal antibodies. *E. coli* ClpP was purified and used to innoculate rabbits (J.M. Flanagan and J. Shanklin, unpublished results). For immunopurification, antigens were immobilized on Affigel-15 (Bio-Rad), and the insoluble proteins first were solubilized in 100 mM sodium phosphate, pH 7.5, containing 3 M guanidine-HCl. Monospecific antibodies were purified from the serum by adsorption to and elution from these columns using 50 mM sodium phosphate, 150 mM NaCl (PBS) as the load and wash buffer, and PBS with 4 M $MgCl_2$ as the elution buffer; the resulting antibodies were desalted into PBS. Preimmune and nonimmune antibodies were purified using immobilized protein G. Immunoprecipitations were performed using *Staphylococcus aureus* cells according to Colbert et al. (1983).

Recombinant Pea ClpC Purification

SG1147 cells expressing pClpCmat were suspended in 40 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT, 0.1% (w/v) Triton X-100, pH 7.5, and lysed using a French pressure cell. The lysate was clarified by centrifugation at 13,000*g* for 30 min. The supernatant fraction was loaded onto a DEAE Fractogel 650 column (E. Merck, Gibbstown, NJ) and eluted with a 0- to 500-mM NaCl gradient. Fractions enriched for ClpC were identified and pooled, dialyzed into 10 mM NaK phosphate, 100 mM KCl, 1 mM DTT, pH 7.5, and applied to an HPLC hydroxyapatite column (Pentax, Tokyo, Japan) equilibrated with 1 mM NaK phosphate, pH 7.5. Proteins were eluted with a 1- to 400-mM NaK phosphate gradient, 1 mM DTT, pH 7.5, and the fractions containing apparently homogeneous ClpC were pooled.

Clp Assays

The fluorometric assay for ClpP was performed according to Woo et al. (1989), using *N*-succinyl-Leu-Tyr-amidomethylcoumarin, a substrate specifically cleaved by *E. coli* ClpP, in 40 mM Tris-HCl, 1 mM DTT, pH 7.5. Caseinolytic activity was monitored by the release of ^3H into TCA-soluble counts from ^3H-methylcasein, using the assay of Katayama-Fujimura et al. (1987).

Immunolocalization and Electron Microscopy

Leaf tissue from 28- to 48-day-old Arabidopsis plants was fixed for 2 hr at room temperature in 0.1% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde in 25 mM Pipes buffer, pH 7.2. The tissue was washed once in 50 mM Pipes, pH 7.2, and then dehydrated in a graded series of ethanol for 20 min each in 30, 50, and 70% ethanol. After an additional 30 min in fresh 70% ethanol, samples were infiltrated for 1 hr with a 1:1 mixture of 70% ethanol and London Resin (LR) White (London Resin Co., Basingstoke, Hampshire, UK) and then for 1 hr with a 1:2 mixture of 70% ethanol and LR White, and samples were then placed in 100% LR White overnight at 4°C. After three more changes in fresh LR White resin, samples were polymerized at 55°C for 24 hr. Ultrathin sections (silver–gold, ~70 to 90 nm) of leaf tissue were collected on nickel grids coated with Formvar (Electron Microscopy Services, Fort Washington, PA). The grids were incubated for 15 min in TBST (20 mM Tris, pH 7.2, 150 mM NaCl, 0.5% Tween) containing 5% nonfat dry milk and 0.1% BSA and then for an additional 15 min in TBST containing 10% normal goat serum. The sections were labeled with 1:5 dilutions of the primary antibodies in TBST and 10% goat serum overnight at 4°C. After the grids were washed five times for 5 min in TBST, they were incubated for 1 hr with TBST and 10% goat serum, containing a 1:20 dilution of 15-nm colloidal gold conjugated to goat anti-rabbit IgG. Grids were washed three times for 5 min in TBST and then three times for 5 min in distilled water. Sections then were poststained in an aqueous 2% (w/v) uranyl acetate solution and viewed with an electron microscope (model 410; Philips, Mahwah, NJ).

Isolation of Chloroplast and Chloroplast Subfractions

Arabidopsis chloroplasts were isolated by Percoll gradient centrifugation (Kunst et al., 1988) and lysed by osmotic shock into 10 mM Hepes-KOH, 5 mM $MgCl_2$, pH 7.5. The total lysate was divided into soluble and membrane fractions by centrifugation at 3000*g* for 5 min.

The supernatant was further clarified by centrifugation at 250,000*g* for 1 hr. A total membrane fraction was washed in 1 M NaBr to remove loosely associated proteins. Thylakoids were lysed by two freeze–thaw cycles, and lumen proteins were recovered after separation of the thylakoid membranes by centrifugation at 3000*g* for 5 min and further clarified by centrifugation at 250,000*g* for 1 hr.

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